Notice to Comply	Application No. 10/776,521	Applicant(s) Flechtner et al.	
	Examiner Benjamin Blumel	Art Unit 1648	

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☐ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☒ 7. Other: The disclosure and claims are missing numbers SEQ ID NO:s, see attached Action under Sequence Compliance.

Applicant Must Provide:

☐ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☐ An initial or substitute paper copy of the "Sequence Listing", **as well as an amendment specifically directing its entry into the application.**

☐ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

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Express Mail No EV 654 849 573 US
Date Mailed: April 10, 2006
Serial No. 10/776,521 Filed February 12, 2004
Inventor Flechtner et al.
For HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES

1. Amendment under 37 C.F.R. § 1.111
and Response to Restriction Requirement Under 37 C.F.R. § 1.142;
2. Peition for Extension of Time for one (1) month (in duplicate);
3. Amendment Fee Transmittal (in duplicate);
4. Information Disclosure Statement Under 37 C.F.R. § 1.56 and § 1.97;
5. List of References Cited;
6. Copies of References A01, B01 to B17 and C01 to C113.

File no.: 8449-405-999 (CAM 708584-999406) Sender: Adriane M. Antler / Richard M. Enmon

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LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)	ATTY. DOCKET NO. 8449-405-999	APPLICATION NO. 10/776,521
	APPLICANT Fletchner et al.	
	FILING DATE February 12, 2004	ART UNIT 1642

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	PAGES, COLUMNS, LINES, WHERE RELEVANT PASSAGES OR RELEVANT FIGURES APPEAR
	A01	10/170,738	06/13/02	Rothman et al.	
	A02	2003/0166530	09/04/03	Rothman et al.	
	A03	2004/0043419	03/04/04	Rothman et al.	
	A04	2005/0214312	09/29/05	Fletchner et al.	
	A05	5,348,945	09/20/94	Berberian et al.	
	A06	5,498,538	03/12/96	Kay et al.	
	A07	5,541,109	07/30/96	Searfoss	
	A08	5,750,119	06/12/98	Srivastava	
	A09	5,837,251	11/17/98	Srivastava	
	A10	5,935,576	08/10/99	Srivastava	
	A11	5,961,979	10/05/99	Srivastava	
	A12	5,962,262	10/5/1999	Hillman et al.	
	A13	5,985,270	11/16/99	Srivastava	
	A14	5,997,873	12/07/99	Srivastava	
	A15	6,017,540	01/25/00	Srivastava et al.	
	A16	6,030,618	02/29/00	Srivastava	
	A17	6,048,530	04/11/00	Srivastava	
	A18	6,127,393	10/3/2000	Fernandex-Pol	
	A19	6,663,868	12/16/2003	Rothman et al.	

FOREIGN PATENT DOCUMENTS

		FOREIGN PATENT DOCUMENT COUNTRY CODE, NUMBER, KIND CODE (IF KNOWN)	DATE	NAME	PAGES, COLUMNS, LINES, WHERE RELEVANT PASSAGES OR RELEVANT FIGURES APPEAR	T
	B01	EP 0 538 952	04/28/93	Yeda Research and Development Co. Ltd.		
	B02	WO 89/04871	06/01/89	Imperial Cancer Research Technology Limited		
	B03	WO 93/17712	09/16/93	Biocine Scalvo Spa		
	B04	WO 94/11513	05/26/94	Medical Research Council		

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

06-14-02 10/170738 A

U.S. DEPARTMENT OF COMMERCE
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UTILITY PATENT APPLICATION TRANSMITTAL LETTER UNDER 37 C.F.R. 1.53(b)	ATTORNEY DOCKET NO 11746/461021

Address to:
Assistant Commissioner for Patents
Washington D.C. 20231
Box Patent Application

Transmitted herewith for filing is the patent application of

Inventor(s): **James E. ROTHMAN, F. Ulrich HARTL, Mee H. HOE, Alan HOUGHTON, Yoshizumi TAKEUCHI and Mark MAYHEW**

For : **HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES**

Enclosed are:

1. **1** sheet of title page with abstract, **43** sheets of specification, **5** sheets of claims (as originally filed) and **6** sheets of amended claims.

2. **5** sheet of drawings.

3. Declaration and Power of Attorney (copy from prior application (37 CFR 1.63(d)) (See paragraph 5 below).

4. Incorporation by Reference. The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under paragraph 4 above is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

5. Power of Attorney by Assignee of Entire Interest (Revocation of Prior Powers and Appointment of New Power) (copy from prior application Serial No. 09/011,645).

6. Other enclosures:

   1. Return Receipt Postcard

7. Continuing application information:

   This application is a continuation of application serial number 09/552,868, filed April 20, 2000, which is a divisional of 09/011,645 filed February 13, 1998, which is a national phase filing of PCT/US96/13363 under 35 U.S.C. § 371, which claims priority of provisional application nos. 60/002,490 and 60/002,479, both filed August 18, 1995.

8. Applicant is a small entity and is entitled to small entity status.

9. The filing fee has been calculated as shown below:

	NUMBER FILED	NUMBER EXTRA*	RATE ($)	FEE ($)
BASIC FEE				740 00
TOTAL CLAIMS	98 - 20 =	78	18 00	1,404 00
INDEPENDENT CLAIMS	5 - 3 =	2	84 00	168 00
MULTIPLE DEPENDENT CLAIM PRESENT			280 00	280 00
*Number extra must be zero or larger			TOTAL	2,592 00
If applicant is a small entity under 37 C F R §§ 1 9 and 1.27, then divide total fee by 2, and enter amount here			SMALL ENTITY TOTAL	1,296 00

10. Please charge the required application filing fee of **$1296.00** to the deposit account of **Kenyon & Kenyon**, deposit account number **11-0600**.

11. The Commissioner is hereby authorized to charge payment of the following fees, associated with this communication or arising during the pendency of this application, or to credit any overpayment to deposit account number **11-0600**:

A. Any additional filing fees required under 37 C.F.R. § 1.16;

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D. Any additional document supply fees under 37 C.F.R. § 1.19;

E. Any additional post-patent processing fees under 37 C.F.R. § 1.20; or

F. Any additional miscellaneous fees under 37 C.F.R. § 1.21.

12. A copy of this sheet is enclosed.

Dated: June 13, 2002

By: _____
Lawrence P. Casson (Reg. No. 46,606)

Description

HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES

The invention described herein was made in the course of work under NIH Core Grant No. CA 08748. The United States government may have certain rights in this invention.

Introduction

The present invention relates to methods and compositions for inducing an immune response in a subject, wherein the subject is administered an effective amount of at least one heat shock protein in combination with one or more defined target antigens. These methods and compositions may be used in the treatment of infectious diseases and cancers.

Background of the Invention

Heat shock proteins were originally observed to be expressed in increased amounts in mammalian cells which were exposed to sudden elevations of temperature, while the expression of most cellular proteins is significantly reduced. It has since been determined that such proteins are produced in response to various types of stress, including glucose deprivation. As used herein, the term "heat shock protein" will be used to encompass both proteins that are expressly labeled as such as well as other stress proteins, including homologs of such proteins that are expressed constitutively (i.e., in the absence of stressful conditions). Examples of heat shock proteins include BiP (also referred to as grp78), hsp/hsc70, gp96 (grp94), hsp60, hsp40 and hsp90.

Heat shock proteins have the ability to bind other proteins in their non-native states, and in particular to bind nascent peptides emerging from ribosomes or extruded into the endoplasmic reticulum. Hendrick and Hartl., *Ann. Rev. Biochem.* 62:349-384 (1993); Hartl., *Nature* 381:571-580 (1996). Further, heat shock proteins have been shown to play

an important role in the proper folding and assembly of proteins in the cytosol, endoplasmic reticulum and mitochondria; in view of this function, they are referred to as "molecular chaperones". Frydman et al., *Nature* 370: 111-117 (1994); Hendrick and Hartl., *Ann. Rev. Biochem.* 62:349-384 (1993); Hartl, *Nature* 381:571-580 (1996).

For example, the protein BiP, a member of a class of heat shock proteins referred to as the hsp70 family, has been found to bind to newly synthesized, unfolded μ immunoglobulin heavy chain prior to its assembly with light chain in the endoplasmic reticulum. Hendershot et al., *J. Cell Biol.* 104:761-767 (1987). Another heat shock protein, gp96, is a member of the hsp90 family of stress proteins which localize in the endoplasmic reticulum. Li and Srivastava, *EMBO J.* 12:3143-3151 (1993); Mazzarella and Green, *J. Biol. Chem.* 262:8875-8883 (1987). It has been proposed that gp96 may assist in the assembly of multi-subunit proteins in the endoplasmic reticulum. Wiech et al., *Nature* 358:169-170 (1992).

It has been observed that heat shock proteins prepared from tumors in experimental animals were able to induce immune responses in a tumor-specific manner; that is to say, heat shock protein purified from a particular tumor could induce an immune response in an experimental animal which would inhibit the growth of the same tumor, but not other tumors. Srivastava and Maki, 1991, *Curr. Topics Microbiol.* 167:109-123 (1991). The source of the tumor-specific immunogenicity has not been confirmed. Genes encoding heat shock proteins have not been found to exhibit tumor-specific DNA polymorphism. Srivastava and Udono, *Curr. Opin. Immunol.* 6:728-732 (1994). High resolution gel electrophoresis has indicated that gp96 may be heterogeneous at the molecular level. Feldweg and Srivastava, *Int. J. Cancer* 63: 310-314 (1995). Evidence suggests that the source of heterogeneity may be populations of small peptides adherent to the heat shock protein, which may number in the hundreds. *Id.* It has been proposed that a wide diversity of peptides

adherent to tumor-synthesized heat shock proteins may render such proteins capable of eliciting an immune response in subjects having diverse HLA phenotypes, in contrast to more traditional immunogens which may be somewhat HLA-restricted in their efficacy. *Id.*

Recently, Nieland et al. (*Proc. Natl. Acad. Sci. U.S.A.* 93:6135-6139 (1996)) identified an antigenic peptide containing a cytotoxic T lymphocyte (CTL) vesicular stomatitis virus (VSV) epitope bound to gp96 produced by VSV-infected cells. Neiland's methods precluded the identification of any additional peptides or other compounds which may also have bound to gp96, and were therefore unable to further characterize higher molecular weight material which was bound to gp96 and detected by high pressure liquid chromatography.

It has been reported that a synthetic peptide comprising multiple iterations of NANP (Asp Ala Asp Pro) malarial antigen, chemically cross-linked to glutaraldehyde-fixed mycobacterial hsp65 or hsp70, was capable of inducing antibody formation (i.e., a humoral response) in mice in the absence of any added adjuvant; a similar effect was observed using heat shock protein from the bacterium *Escherichia coli*. Del Guidice, *Experientia* 50:1061-1066 (1994); Barrios et al., *Clin. Exp. Immunol.* 98:224-228 (1994); Barrios et al., *Eur. J. Immunol.* 22:1365-1372 (1992). Cross-linking of synthetic peptide to heat shock protein and possibly glutaraldehyde fixation was required for antibody induction. Barrios et al., *Clin. Exp. Immunol.* 98:229-233.

It has now been discovered, according to the present invention, that heat shock protein may be combined with target antigen and used to induce an immune response which includes a cytotoxic cellular component, i.e., a cellular response.

Summary of the Invention

The present invention relates to methods and compositions for inducing an immune response in a subject, wherein

at least one heat shock protein in combination with one or more defined target antigens is administered to the subject.

Unlike prior disclosures relating to heat shock protein associated with an undefined population of potential antigens which have been restricted, in their immunogenic effect, to a single tumor, the present invention provides for methods and compositions which combine heat shock protein with a defined target antigen which may be selected on the basis that it is immunogenic in diverse occurrences of a neoplastic or infectious disease, or because it has been identified, in an individual instance, as being particularly immunogenic. Further, because the use of one or more defined target antigen permits more control over the immune response elicited, it may avoid the induction of an undesirable immune response.

In alternative embodiments of the invention, the target antigen may be either (i) an antigen which itself binds to the heat shock protein; or (ii) a hybrid antigen comprising an immunogenic domain as well as a heat shock protein-binding domain. The immunogenic domain may be an entire protein or peptide antigen, or may be only a portion of the selected antigen, for example a selected epitope of the antigen. In specific, nonlimiting embodiments of the invention, the heat shock protein binding domain may comprise a peptide having the sequence:

His Trp Asp Phe Ala Trp Pro Trp [SEQ. ID NO. 1]

The present invention provides for methods of administering such heat shock protein/target antigen compositions comprising (i) combining one or more heat shock protein with one or more target antigens *in vitro*, under conditions wherein binding of target antigen to heat shock protein occurs to form a target antigen/heat shock protein complex; and (ii) administering the target antigen, bound to heat shock protein, in an effective amount to a subject in need of such treatment.

Alternatively, heat shock protein/target antigen combinations of the invention may be administered to a

subject by introducing nucleic acid encoding the heat shock protein and the target antigen into the subject such that the heat shock protein and target antigen bind *in situ*.

Brief Description of the Drawings

Fig. 1 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

Fig. 2 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

Fig. 3 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

Fig. 4 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

Figs. 5A and 5B shows the results of control experiments in which hybrid peptide or Ova-peptide and heat shock protein were administered individually to EL4 cells;

Fig. 6 shows co-elution of hybrid peptides and heat shock proteins from a column, demonstrating binding of the polypeptides to the heat shock protein;

Fig. 7 shows the co-elution of ^{125}I-OVA-BiP with BiP in the presence and absence of ATP;

Fig. 8 shows the killing efficacy of T-cells primed with various combinations of antigens and heat shock proteins on EL4 cells pulsed with antigen; and

Fig. 9 shows the killing efficacy of T-cells primed with various concentrations of antigens and heat shock proteins on EG7 lymphoma cells.

Detailed Description of the Invention

For purposes of clarity of description, and not by way of limitation, the detailed description is divided into the following subsections:

(i) heat shock proteins;

(ii) target antigens; and
(iii) methods of administration.

Heat Shock Proteins

The term "heat shock protein", as used herein, refers to any protein which exhibits increased expression in a cell when the cell is subjected to a stress. In preferred nonlimiting embodiments, the heat shock protein is originally derived from a eukaryotic cell; in more preferred embodiments, the heat shock protein is originally derived from a mammalian cell. For example, but not by way of limitation, heat shock proteins which may be used according to the invention include BiP (also referred to as grp78), hsp/hsc70, gp96(grp94), hsp60, hsp40, and hsp90. Especially preferred heat shock proteins are BiP, gp96, and hsp70, as exemplified below. Naturally occurring or recombinantly derived mutants of heat shock proteins may also be used according to the invention. For example, but not by way of limitation, the present invention provides for the use of heat shock proteins mutated so as to facilitate their secretion from the cell (for example having mutation or deletion of an element which facilitates endoplasmic reticulum recapture, such as KDEL or its homologs; such mutants are described in concurrently filed PCT Application No. _____ (Attorney Docket No. MSK.P-018), which is incorporated herein by reference.

For embodiments of the invention wherein heat shock protein and target antigen are directly administered to the subject in the form of a protein/peptide complex, the heat shock protein may be prepared, using standard techniques, from natural sources, for example as described in Flynn et al., *Science* 245: 385-390 (1989), or using recombinant techniques such as expression of a heat shock encoding vector in a suitable host cell such as a bacterial, yeast or mammalian cell. If pre-loading of the heat shock protein with peptides from the host organism is a concern, the heat shock protein can be incubated with ATP and then repurified.

Nonlimiting examples of methods for preparing recombinant heat shock proteins are set forth below.

A nucleic acid encoding a heat shock protein may be operatively linked to elements necessary or desirable for expression and then used to express the desired heat shock protein as either a means to produce heat shock protein for use in a protein vaccine or, alternatively, in a nucleic acid vaccine. Elements necessary or desirable for expression include, but are not limited to, promoter/enhancer elements, transcriptional start and stop sequences, polyadenylation signals, translational start and stop sequences, ribosome binding sites, signal sequences and the like. For example, but not by way of limitation, genes for various heat shock proteins have been cloned and sequenced, including, but not limited to, gp96, human: Genebank Accession No. X15187; Maki et al., *Proc. Nat'l Acad. Sci.* 87: 5658-5562 (1990), mouse: Genebank Accession No. M16370; Srivastava et al., *Proc. Nat'l Acad. Sci.* 84:3807-3811 (1987)); BiP, mouse: Genebank Accession No. U16277; Haas et al., Proc. Nat'l Acad. Sci. U.S.A. 85: 2250-2254 (1988), human: Genebank Accession No. M19645; Ting et al., DNA 7: 275-286 (1988); hsp70, mouse: Genebank Accession No. M35021; Hunt et al., *Gene* 87: 199-204 (1990), human: Genebank Accession No. M24743; Hunt et al., Proc. Nat'l Acad. Sci. U.S.A. 82: 6455-6489 (1995); and hsp40 human: Genebank Accession No. D49547; Ohtsuka K., *Biochem Biophys. Res. Commun.* 197: 235-240 (1993).

Target Antigens

A target antigen, according to the invention, may be either (i) an antigen which itself binds to the heat shock protein; or (ii) a hybrid antigen comprising an immunogenic domain as well as a heat shock protein-binding domain. Thus, the target antigen serves at least two functions, namely (I) it contains an epitope capable of inducing the desired immune response; and (ii) it is capable of physically binding to its partner heat shock protein. Of note, the term "physically binds" indicates that the target antigen and

heat shock protein exhibit a physical interaction which permits the adherence of one to the other for at least a transient period of time; of note, the binding need not, and in most embodiments of the invention should not, be irreversible.

In certain embodiments, an antigen capable of inducing the desired immune response may be found to be inherently capable of binding to a partner heat shock protein. In other embodiments, it may be necessary or desirable to link an immunogenic antigen to one or more other compounds so as to create a hybrid antigen which contains both an immunogenic domain as well as a heat shock protein binding domain. In such circumstances, a compound which is, itself, an immunogenic antigen may be linked to a compound which is, itself, capable of binding to a heat shock protein. Alternatively, the linkage of two or more compounds which individually lack either functionality may give rise to the desired immunogenic and binding characteristics.

The term "antigen" as used herein, refers to a compound which may be composed of amino acids, carbohydrates, nucleic acids or lipids individually or in any combination.

The term "target antigen", as used herein, refers to a compound which binds to one or more heat shock proteins and which is representative of the immunogen toward which an immune response is desirably directed. For example, where the immunogen is an influenza virus, the target antigen may be a peptide fragment of the matrix protein of the influenza virus. As used herein, the term "immunogen" is applied to the neoplastic cell, infected cell, pathogen, or component thereof, towards which an immune response is to be elicited, whereas the target antigen is a portion of that immunogen which can provoke the desired response and which inherently or through engineering binds to one or more heat shock proteins. In particular, the target antigen is selected to elicit an immune response to a particular disease or pathogen, including peptides obtained from MHC molecules,

mutated DNA gene products, and direct DNA products such as those obtained from tumor cells.

While the invention may be applied to any type of immunogen, immunogens of particular interest are those associated with, derived from, or predicted to be associated with a neoplastic disease, including but not limited to a sarcoma, a lymphoma, a leukemia, or a carcinoma, and in particular, with melanoma, carcinoma of the breast, carcinoma of the prostate, ovarian carcinoma, carcinoma of the cervix, colon carcinoma, carcinoma of the lung, glioblastoma, astrocytoma, etc. Further, mutations of tumor suppressor gene products such as p53, or oncogene products such as ras may also provide target antigens to be used according to the invention.

In further embodiments, the immunogen may be associated with an infectious disease, and, as such, may be a bacterium, virus, protozoan, mycoplasma, fungus, yeast, parasite, or prion. For example, but not by way of limitation, the immunogen may be a human papilloma virus (see below), a herpes virus such as herpes simplex or herpes zoster, a retrovirus such as human immunodeficiency virus 1 or 2, a hepatitis virus, an influenza virus, a rhinovirus, respiratory syncytial virus, cytomegalovirus, adenovirus, *Mycoplasma pneumoniae*, a bacterium of the genus *Salmonella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Vibrio*, *Mycobacterium*, amoeba, a malarial parasite, *Trypanosoma cruzi*, etc.

Immunogens may be obtained by isolation directly from a neoplasm, an infected cell, a specimen from an infected subject, a cell culture, or an organism culture, or may be synthesized by chemical or recombinant techniques. Suitable antigenic peptides, particularly for use in a hybrid antigen, for use against viruses, bacteria and the like can be designed by searching through their sequences for MHC class I restricted peptide epitopes containing HLA binding sequences such as but not limited to HLA-A2 peptide binding sequences:

Xaa(Leu/Met)XaaXaaXaa(Val/Ile/Leu/Thr)XaaXaa(Val/Leu) [SEQ ID No. 2], Rammensee et al., *Immunogenetics* 41: 178-223 (1995), Xaa(Leu/Met)XaaXaaXaaXaaXaaXaaVal [SEQ ID No. 3], Tarpey, et al *Immunology* 81: 222-227 (1994), Xaa(Val/Gln)XaaXaaXaaXaaXaaXaaLeu [SEQ ID No. 28] Barouch et al., *J. Exp. Med.* 182: 1847-1856 (1995).

It may also be desirable to consider the type of immune response which is desired. For example, under certain circumstances, a humoral immune response may be appropriate. In other cases, and indeed where an immune response directed toward neoplastic cells or infected cells is sought to be elicited, a cellular immune response is particularly desirable. Accordingly, particular epitopes associated with the activation of B cells, T helper cells, or cytotoxic T cells may be identified and selected for incorporation into the target antigen.

It may also be desirable to utilize target antigen associated with an autoimmune disease or allergy. Such a target antigen may be administered, together with one or more heat shock proteins, in an amount sufficient to be tolerogenic or to inhibit a pre-existing immune response to the target antigen in a subject. The amount of heat shock protein required to inhibit the immune response is expected to be substantially greater than the amount required for stimulation.

Although the size of target antigen may vary depending upon the heat shock protein used, in nonlimiting embodiments of the invention, the target antigen may be the size of a peptide having between 4 and 500 of amino acid residues, and preferably be the size of a peptide having between 4 and 100, most preferably 7 and 20 amino acid residues. As such, it may be desirable to produce a fragment of an immunogen to serve as a target antigen, or, alternatively, to synthesize a target antigen by chemical or recombinant DNA methods. In some instances, however, an immunogen may, in intact form, serve as a target antigen.

Based on the foregoing considerations, a target antigen may be prepared, and then tested for its ability to bind to heat shock protein. In some instances, binding of target antigen to a particular heat shock protein may be facilitated by the presence of at least one other protein, which may be a heat shock protein.

For example, binding of target antigen to a heat shock protein may be evaluated by labeling the target antigen with a detectable label, such as a radioactive, fluorescent, enzymatic or pigmented label, combining the target antigen with heat shock protein under conditions which would be expected to permit binding to occur, and then isolating the heat shock protein while removing any unbound target antigen, and determining whether any labeled target antigen had adhered to the heat shock protein. As a specific example, and not by way of limitation, the ability of a target antigen to bind to BiP heat shock protein may be evaluated by combining 2 µg BiP with up to about 1150 pmole of radioactively labeled target antigen in buffer containing 50 mM Tris HCl (pH 7.5), 200 mM NaCl, and 1 mM Na_2EDTA, in a final volume of 50 µl, for 30 minutes at 37 degrees Centigrade. Unbound target antigen may then be removed from bound BiP-target antigen by centrifugation at 100g by desalting through a 1 ml Sephadex-G column for 2 minutes. Penefsky, *J. Biol. Chem.* 252:2891 (1977). To prevent binding to the resin, columns may first be treated with 100 µl of bovine serum albumin in the same buffer and centrifuged as above. Bound target antigen may then be quantitated by liquid scintillation counting. See Flynn et al., *Science* 245:385-390 (1989).

Because ATP hydrolysis drives the release of peptides from many known heat shock proteins, the amount of ATPase activity may often be used to quantitate the amount of target antigen binding to heat shock protein. An example of how such an assay may be performed is set forth in Flynn et al., *Science* 245:385-390 (1990).

If a particular immunogen or a fragment thereof does not satisfactorily bind to a heat shock protein, then that immunogen or fragment may be linked to another compound so as to create a heat shock protein-binding domain thereby constructing a hybrid antigen. The heat shock protein-binding domain is selected so that the hybrid peptide will bind *in vitro* to a heat shock protein such as BiP, hsp70, gp96, or hsp90, alone or in combination with accessory heat shock proteins such as hsp40, or hsp60. Peptides which fulfill this criterion may be identified by panning libraries of antigens known to bind well to one or more heat shock proteins as described in Blond-Elguindi et al., *Cell* 75:717-728 (1993). Using this technique, Blond-Elguindi have concluded that the heat shock protein BiP recognizes polypeptides that contain a heptameric region having the sequence

Hy(Trp/X)HyXHyXHy

where Hy represents a hydrophobic amino acid residue, particularly tryptophan, leucine or phenylalanine, and X is any amino acid. High affinity heat-shock protein-binding sequences incorporating this motif include:

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]; and

Phe Trp Gly Leu Trp Pro Trp Glu [Seq. ID No. 4].

Other heat shock protein binding motifs have also been identified. For example, Auger et al. *Nature Medicine* 2:306-310 (1996) have identified two pentapeptide binding motifs

Gln Lys Arg Ala Ala [SEQ ID No. 5] and

Arg Arg Arg Ala Ala [Seq. ID No. 6]

in HLA-DR types associated with rheumatoid arthritis which bind to heat shock proteins. Heat shock binding motifs have also been identified as consisting of seven to fifteen residue long peptides which are enriched in hydrophobic amino acids. Flynn et al., *Science* 245: 385-390 (1989); Gragerov et al., *J. Molec. Biol.* 235: 848-854 (1994).

The hybrid antigen of the invention incorporates one immunogenic domain and one heat shock protein-binding

domain, optionally separated by a short peptide linker. The hybrid peptide of the invention may be synthesized using chemical peptide synthesis methods or it can be synthesized by expression of a nucleic acid construct containing linked sequences encoding the antigenic and heat shock protein-binding domains. One suitable technique utilizes initial separate PCR amplification reactions to produce separate DNA segments encoding the two domains, each with a linker segment attached to one end, followed by fusion of the two amplified products in a further PCR step. This technique is referred to as linker tailing. Suitable restriction sites may also be engineered into regions of interest, after which restriction digestion and ligation is used to produce the desired hybrid peptide-encoding sequence.

Methods of Administration

The heat shock protein/target antigen combinations of the invention may be administered to a subject using either a protein-based or nucleic acid vaccine, so as to produce, in the subject, an amount of heat shock protein/target antigen complex which is effective in inducing a therapeutic immune response in the subject.

The subject may be a human or nonhuman subject.

The term "therapeutic immune response", as used herein, refers to an increase in humoral and/or cellular immunity, as measured by standard techniques, which is directed toward the target antigen. Preferably, but not by way of limitation, the induced level of humoral immunity directed toward target antigen is at least four-fold, and preferably at least 16-fold greater than the levels of the humoral immunity directed toward target antigen prior to the administration of the compositions of this invention to the subject. The immune response may also be measured qualitatively, wherein by means of a suitable *in vitro* assy or *in vivo* an arrest in progression or a remission of neoplastic or infectious disease in the subject is considered to indicate the induction of a therapeutic immune response.

Specific amounts of heat shock protein/target antigen administered may depend on numerous factors including the immunogenicity of the particular vaccine composition, the immunocompetence of the subject, the size of the subject and the route of administration. Determining a suitable amount of any given composition for administration is a matter of routine screening.

In specific nonlimiting embodiments of the invention, it may be desirable to include more than one species of heat shock protein, and/or more than one target antigen, in order to optimize the immune response. Such an approach may be particularly advantageous in the treatment of cancer or in the treatment of infections characterized by the rapid development of mutations that result in evasion of the immune response.

In other specific nonlimiting embodiments of the invention, in order to promote binding among members of each heat shock protein/target antigen pair, the ratio of heat shock protein to target antigen may preferably be 1:2 to 1:200. Higher relative levels of antigen are suitable to enhance binding to the heat shock protein.

According to still further specific but nonlimiting embodiments of the invention, the target antigen is not chemically cross-linked to the heat shock protein.

Compositions comprising target antigen/heat shock protein as set forth above are referred to herein as "vaccines". The term vaccine is used to indicate that the compositions of the invention may be used to induce a therapeutic immune response.

A vaccine composition comprising one or more heat shock proteins and one or more target antigens in accordance with the invention may be administered cutaneously, subcutaneously, intravenously, intramuscularly, parenterally, intrapulmonarily, intravaginally, intrarectally, nasally or topically. The vaccine composition may be delivered by injection, particle bombardment, orally or by aerosol.

Incubation of heat shock proteins in solution with the target antigen is sufficient to achieve loading of the antigen onto the heat shock protein in most cases. It may be desirable in some cases, however, to add agents which can assist in the loading of the antigen.

Incubation with heating of the heat shock protein with the target antigen will in general lead to loading of the antigen onto the heat shock protein. In some cases, however, it may be desirable to add additional agents to assist in the loading. For example, hsp40 can facilitate loading of peptides onto hsp70. Minami et al., *Gen. Biol Chem.* 271: 19617-19624 (1996). Denaturants such as guanidinium HCl or urea can be employed to partially and reversibly destabilize the heat shock protein to make the peptide binding pocket more accessible to the antigen.

Vaccine compositions in accordance with the invention may further include various additional materials, such as a pharmaceutically acceptable carrier. Suitable carriers include any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

The vaccine composition of the invention may also include suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions may be in the form of liquid or lyophilized or otherwise dried formulations and may include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and

ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g. glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexing with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc. or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. The choice of compositions will depend on the physical and chemical properties of the vaccine. For example, a product derived from a membrane-bound form of a protein may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhances for various routes of administration, including intramuscular, parenteral, pulmonary, nasal and oral.

As an alternative to direct administration of the heat shock protein and target antigen, one or more polynucleotide constructs may be administered which encode heat shock protein and target antigen in expressible form. The expressible polynucleotide constructs are introduced into cells in the subject using *ex vivo* or *in vivo* methods.

Suitable methods include injection directly into tissue and tumors, transfecting using liposomes (Fraley et al., *Nature* 370:111-117 (1980)), receptor-mediated endocytosis (Zatloukal, et al., *Ann. NY Acad. Sci.* 660:136-153 (1992)); particle bombardment-mediated gene transfer (Eisenbraun et al., *DNA & Cell Biol.* 12:792-797 (1993)) and transfection using peptide presenting bacteriophage. Barry et al. *Nature Medicine* 2: 299-305 (1996). The polynucleotide vaccine may also be introduced into suitable cells *in vitro* which are then introduced into the subject.

To construct an expressible polynucleotide, a region encoding the heat shock protein and/or target antigen is prepared as discussed above and inserted into a mammalian expression vector operatively linked to a suitable promoter such as the SV40 promoter, the cytomegalovirus (CMV) promoter or the Rous sarcoma virus (RSV) promoter. The resulting construct may then be used as a vaccine for genetic immunization. The nucleic acid polymer(s) could also be cloned into a viral vector. Suitable vectors include but are not limited to retroviral vectors, adenovirus vectors, vaccinia virus vectors, pox virus vectors and adenovirus-associated vectors. Specific vectors which are suitable for use in the present invention are pCDNA3 (In-Vitrogen), plasmid AH5 (which contains the SV40 origin and the adenovirus major late promoter). pRC/CMV (InVitrogen), pCMU II (Paabo et al., *EMBO J.* 5:1921-1927 (1986)), pZip-Neo SV (Cepko et al., *Cell* 37:1053-1062 (1984)) and pSRα (DNAX, Palo Alto, CA).

EXAMPLE 1

PREPARATION OF HYBRID PEPTIDES

Hybrid peptides containing a BiP-binding domain (His-Trp-Asp-Phe-Ala-Trp-Pro-Trp; SEQ ID NO: 1) and an OVA antigenic domain (Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu; SEQ ID NO:7) separated by a tripeptide linker (gly-ser-gly) were synthesized. Peptides were produced in both orientations, OVA-BiP-binding domain and BiP-binding domain OVA as follows:

Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Gly-Ser-Gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID NO: 8]
and
His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-Gly-Ser-Gly-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu [SEQ ID NO. 9].

EXAMPLE 2

Purified mouse cytosolic hsp70 was prepared from *E. coli* DH5α cells transformed with pMS236 encoding mouse cytosolic hsp70. The cells were grown to an optical density (600 nm) of 0.6 at 37°C, and expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation 2 to 5 hours post induction and the pellets were resuspended to 20 mL with Buffer A (20 mM Hepes pH 7.0, 25 mM KCl, 1 mM DTT, 10 mM $(NH_4)_2SO_4$, 1mM PMSF). The cells were lysed by passing three times through a French press. The lysate was cleared by a low speed spin, followed by centrifugation at 100,000 X G for 30 minutes. The cleared lysate was applied to a Pharmacia XK26 column packed with 100 mL DEAE Sephacel and equilibrated with Buffer A at a flow rate of 0.6 cm/min. The column was washed to stable baseline with Buffer A and eluted with Buffer A adjusted to 175 mM KCl. The eluate was applied to a 25 mL ATP-agarose column (Sigma A2767), washed to baseline with Buffer A, and eluted with Buffer A containing 1 mM MgATP preadjusted to pH 7.0. EDTA was added to the eluate to a final concentration of 2 mM. The eluate which contained essentially pure hsp70 was precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitate was resuspended in Buffer A containing 1 mM $MgCl_2$ and dialyzed against the same buffer with multiple changes. The purified hsp70 was frozen in small aliquots at -70°C.

EXAMPLE 3

The purified hsp70 was combined with the synthesized peptides and used for immunization. To form the hsp70/peptide mixtures, approximately 15 ug (21.5 uM) hsp70 was

combined with 5 ug of Ova-peptide (0.5 mM, SEQ. ID. NO: 5) or 10 ug (0.5 mM) hybrid peptide (SEQ. ID NOS: 6 and 7) were mixed on ice to a final volume of 10 µl in Buffer B (final concentration: 20 mM Hepes pH 7.0, 150 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ and 2 mM MgATP, pH 7.0). The mixtures were incubated for 30 minutes at 37°C and then used for *in vivo* immunizations.

C57BL/6 mice were immunized intradermally once a week for a total of two weeks with 10 µL of one of the mixtures described above or with a mixture of TiterMax® (Vaxell, Norcross, GA) and Ova-peptide (5 µg). One week after the second immunization, spleen cells were removed and mononuclear cells (6-8 X 10^7) were cultured with 3 X 10^6 γ-irradiated (3000 rad) stimulator cells. The stimulator cells were obtained from naive mice that had been sensitized *in vitro* with Ova-peptide (10 mg/ml) for 30 minutes at room temperature, washed and irradiated at 3000 rads.

Cytotoxicity of spleen cells from vaccinated mice were assayed on Ova-peptide pulsed EL4 cells in an 18-hour chromium release assay. CTL were generated by culturing *in vivo* immunized spleen cells for 5 days at a concentration of 10^6 cells/mL in RPMI medium, 10% FCS, penicillin, streptomycin and 2 mM L-glutamine, together with 3 X 10^6 γ-irradiated (3,000 rad) stimulator cells/mL. Target cells were prepared by culturing cells for 1 hour in the presence of 250 µCi of ^{51}Cr sodium chromate (DuPont, Boston, MA) in Tris-phosphate buffer, pH 7.4 at 37°C for 60 minutes. After washing, 10^4 ^{51}Cr-labeled target cells were mixed with effector lymphocytes to yield several different effector/target (E/T) ratios and were incubated for 18 hours. Supernatants were harvested and the radioactivity was measured in a gamma counter. Percent specific lysis was calculated as: 100 X[(cpm release by CTL-cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)]. Maximal response was determined by addition of 1% Triton X-100. Spontaneous release of all target in the absence of effector cells was less than 25% of the maximal release.

As shown in Figure 1, the combination of Hsp70 and the hybrid peptide of either orientation (hsp7+BiP-OVA or hsp70+OVA-BiP) evoked a higher immune response as measured by specific lysis of cells than the hsp70 or TiterMax® adjuvant plus Ova-peptide alone.

EXAMPLE 4

The assay of Example 3 was repeated using CTL cell lines which had been maintained by stimulation with irradiated stimulators, syngeneic splenic feeder cells plus T cell growth factors for a period of two weeks. As shown in Fig. 2, the combination of hsp70 and the hybrid peptide of either orientation (hsp70+BiP-OVA or hsp7+OVA-BiP) evoked a higher immune response as measured by specific lysis of cells than the hsp70 or TiterMax® adjuvant plus Ova-peptide alone. Thus, the immune response elicited by the hybrid peptides persisted through additional passages and can be maintained over a period of time.

EXAMPLE 5

The experiment of Example 2 was repeated for the combinations of hsp70 plus BiP-OVA and TiterMax® plus Ova-peptide using only a single immunization one week before removal of the spleen cells. As shown in Fig. 3, the single immunization with either composition was effective in eliciting a cellular immune response.

EXAMPLE 6

The assay of Example 3 was repeated using mixtures of TiterMax® with Ova-peptide or the hybrid peptides of Example 1. As shown in Fig. 4, no significant difference was observed between the Ova-peptide and hybrid peptides demonstrating the specificity of the effect when hybrid peptides are used in association with the heat shock protein.

EXAMPLE 7

Figs. 5A and 5B show the results when the procedure of Example 3 was repeated immunizing the mice with hsp70 alone, OVA-peptide alone, Ova-BiP alone or Bip-Ova alone. As shown, the results in all cases were the same when the cells were pulsed with Ova-peptide (Fig. 5A) and when they had not been pulsed. (Fig. 5B). This demonstrates that the response is the result of the combination of the mixture of the antigen (Ova-peptide or hybrid peptide) and the heat shock protein and not to any of the components individually.

EXAMPLE 8

^{14}C-labeled OVA-BiP was prepared by alkylation of OVA-BiP with ^{14}C-formaldehyde. 0.9 mg of OVA-BiP in 300 uL 10% DMSO/water was added to 175 µl of ^{14}C-formaldehyde (62 µCi) and immediately 50 uL of freshly made up 200 mM $NaCNBH_3$ was added. The reaction was mixed and left at 25°C for 3 hours. The labeled peptide was repurified by reverse phase HPLC on a C-4 column in a 15 minute 0-100% acetonitrile (0.1% TFA) gradient.

The ability of the OVA-BiP peptide to bind to heat shock proteins was measured by incubating 100 µM (5µg) ^{14}C-labeled OVA-BiP with 50 µg of BiP (prepared as in example 11), hsp70 (as prepared in Example 2) or grp96 (prepared as in Example 10) in a final volume of 20 µl of buffer (50 mM Mops, pH 7.2., 200m mM NaCl, 5 mM MgAcetate) at 37°C for 30 minutes. The samples were then spun down (5 minutes in a microfuge) and loaded onto a 17 cm long Sephacryl S-300 column equilibrated in binding buffer (50 mM Mops, pH 7.2., 200 mM NaCl, 5 mM MgAcetate) and fractions were collected dropwise. 50 µl of each ~225 µl fraction was counted in scintillation liquid. 10 µl of each fraction was also run on a 12% SDS-PAGE reducing gel. Fig. 6 shows the radio-activity detected in each fraction eluted from the column, together with the center of the peak of heat shock protein as determined by SDS-PAGE. As shown, a significant amount of radioactivity elutes with BiP and hsp70, thus providing

evidence that the hybrid peptide binds to these two heat shock proteins. The result for gp96 is unclear because the peak at fraction 11 (which may represent an aggregation phenomenon) and the gp96 peak (fraction 14) elute close together on the column used.

EXAMPLE 9

To prepare ^{125}I-OVA-BiP, 250 µCi of monoiodinated Bolton-Hunter reagent was transferred into a stoppered vial and the solvent in which it was dissolved was evaporated with a gentle stream of argon gas. To the dried reagent 222 µL of 4.5 mg/mL OVA-BiP in 100 mM $NaBO_3$, pH 8, 9, 10% DMSO was added. The reaction was mixed and incubated at 25°C for 45 minutes and continued at 4°C for a further hour. The labeled peptide was repurified by reverse phase HPLC on a C-4 column in a 20 minute, 0-100% acetonitrile (0.1% TFA) gradient.

The iodinated OVA-BiP was combined with BiP in substantially the same manner as the heat shock proteins in Example 7, except that since the iodinated peptide was at a very low concentration, 1 µl (approx 32 ng) of labeled peptide was mixed with 5 µg of unlabeled peptide and this was incubated with 50 µg of BiP in 20 µL of binding buffer. To observe ATP-mediated peptide release, ATP was added to a final concentration of 2 mM after the 30 minute incubation and incubated for a further 5 minutes prior to spinning. These samples were run on the same column as above, but equilibrated in binding buffer supplemented with 2 mM ATP.

Fig. 7 shows the elution profile for a mixture of the ^{125}I-OVA-BiP and BiP in the presence and absence of 2 mM ATP. As shown, addition of ATP causes the release of the hybrid peptide from the BiP. This is consistent with the observation that ATP mediates release of bound proteins or polypeptides from heat shock proteins.

EXAMPLE 10

Hybrid peptides for use in a vaccine in accordance with the invention against human papilloma virus are prepared using a peptide synthesizer as follows:

E7 (Type 11)-BiP

Leu-Leu-Leu-Gly-Thr-Leu-Asn-Ile-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 10]

BiP-E7 (Type 11)

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Leu-Leu-Leu-Gly-Thr-Leu-Asn-Ile-Val [SEQ ID No. 11]

E7 (Type 16)-BiP

Leu-Leu-Met-Gly-Thr-Leu-Gly-Ile-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 12]

BiP-E7 (Type 16)

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Leu-Leu-Met-Gly-Thr-Leu-Gly-Ile-Val [SEQ ID No. 13]

E7 (Type 18)-BiP

Thr-Leu-Gln-Asp-Ile-Val-Leu-His-Leu-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 14]

BiP-E7 (Type 18)

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Thr-Leu-Gln-Asp-Ile-Val-Leu-His-Leu [SEQ ID No. 15]

E7.1 (Type 6b)-BiP
Gly-Leu-His-Cys-Tyr-Glu-Gln-Leu-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 16]

BiP-E7.1 (Type 6b)
His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Gly-Leu-His-Cys-Tyr-Glu-Gln-Leu-Val [SEQ ID No. 17]

E7.2 (Type 6b)-BiP
Pro-Leu-Lys-Gln-His-Phe-Gln-Ile-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 18]

BiP-E7.2 (Type 6b)
His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Pro-Leu-Lys-Gln-His-Phe-Gln-Ile-Val [SEQ ID No. 19]

Hybrid polypeptides for use in vaccines against human papilloma virus of other types or proteins from other viruses, bacteria etc can be developed by searching their sequences for MHC class I restricted peptide epitopes containing the HLA-A2 peptide binding motif.

EXAMPLE 11

PREPARATION OF RECOMBINANT GP96

The DNA sequence encoding a wild-type or KDEL-deleted gp96 polypeptide was subcloned from pRc/CMV into the vector pET11a (Novagen). Thus upon expression, mature gp96 could be purified from cell lysates.

Vector construction

PCR amplification of the sequence encoding gp96 (from pRc/CMV) was performed with the following primers. The 5' primer for both wild-type and KDEL-deleted gp96 was complementary to the DNA sequence encoding the amino terminal end of the mature form of gp96 and an Nde I restriction site (CATATG) the ATG of which forms the initiator codon:

5' AGA TAT ACA TAT GGA TGA TGA AGT CGA CGT GG 3'

[SEQ ID No. 20]

The 3' primers were complementary to the DNA sequence of gp96 encoding the carboxyl terminal end of the protein, with the nucleotides encoding the KDEL sequence removed in the primer for the KDEL-deleted variant. Both primers contain a BamH I restriction site (GGATCC) followed by a STOP codon as shown:

Wild-type:

5' TCG GAT CCT TAC AAT TCA TCC TTC TCT GTA GAT TC 3'

[SEQ ID No. 21]

KDEL-deleted:

5' TCG GAT CCT TAC TCT GTA GAT TCC TTT TC 3'

[SEQ ID No. 22]

The PCR products were cut with Nde I and BamH I and ligated into pET11a (Novagen) which had also been cut with these enzymes. The ligation product was used to transform competent BL21 cells. Clones obtained were screened by expression screening.

Expression and Purification

This procedure is identical for wild-type or KDEL-deleted gp96. Two liters of *E. coli* BL21 cells transformed with pET11a containing a sequence coding for either wild-type or KDEL-deleted gp96 were grown in 2xTY medium supplemented with 200 ug/ml ampicillin at 37°C until they reached an absorbance at 600 nm of 0.5-0.6 at which point they were induced by the addition of 1 mM IPTG. The cells were allowed to grow for a further 2-5 hours at 37°C and then they were harvested by 10 minutes centrifugation at 7000 x G. The cell pellet was resuspended in 50mM Hepes pH 7.5, 50mM KCl, 5mM MgAcetate, 20% sucrose, 1mM PMSF and the cells lysed by passing them through the French Press three times. The cell extract was clarified by a one hour spin at 200000 x G and the supernatant retained.

The supernatant was diluted two-fold with cold 50 mM Hepes pH 7.5 and loaded onto a Pharmacia XK26 column containing 50 ml of DE52 anion exchange resin (Whatman) which

had been equilibrated in 50 mM Mops pH 7.4., 10 mM NaCl, 5 mM MgAcetate. The bound protein was eluted in a 0-1000 mM NaCl gradient. Fractions containing gp96 were identified by SDS-PAGE and pooled.

The pooled gp96-containing fractions were diluted two-fold with cold 50 mM Mops pH 7.4 and loaded onto a Pharmacia XK16 column containing 15 mL of hydroxylapatite resin (BioRad) which had been washed with 0.5 M K_2HPO_4 pH 7.2., 50 mM KCl and equilibrated in 10 mM K_2HPO_4 pH 7.2, 50mM KCl. The bound protein was eluted in a 10-500 mM K_2HPO_4 pH 7.2 gradient with the KCl concentration held constant at 50mM. Fractions containing gp96 were identified by SDS-PAGE and pooled.

The pooled gp96-containing fractions were finally loaded onto a Pharmacia XK26 column containing 25ml of phenyl Sepharose (Pharmacia) which had been equilibrated in 50mM Mops pH 7.2, 500mM NaCl and eluted in a 500-0mM NaCl gradient. The fractions containing essentially pure gp96 were pooled, concentrated by filtration and made up to 10% glycerol. The purified gp96 was stored frozen at -80°C.

EXAMPLE 12

Construction of BiP Expression vector and Purification of Recombinant BiP

The DNA sequence encoding the wild-type or KDEL-deleted BiP polypeptide was subcloned from pCDNA3 into the vector pET22 (Novagen), thereby placing it behind and in frame with a DNA sequence that codes for a signal sequence which targets the expressed BiP to the periplasmic space of the bacterial expression host, *E. coli*. Upon transport into the periplasm the signal sequence is removed and thus mature wild-type or KDEL-deleted BiP can be harvested from the periplasm without any contamination by cytosolic hsp70s.

Vector construction:

PCR amplification of the sequence encoding BiP (from pCDNA3) was performed with the following primers. The 5'

primer for both wild-type and KDEL-deleted BiP was complementary to the DNA sequence of BiP encoding the amino terminal end of the mature form of BiP with an Msc I restriction site (TGGCCA) immediately upstream from the initiator ATG codon:

5' AGA TAT GTG GCC ATG GAG GAG GAG GAC AAG 3'

[SEQ ID No. 23]

The 3' primers were complementary to the DNA sequence of BiP encoding the carboxyl terminal end of the protein, with the nucleotides encoding the KDEL sequence removed in the primer for the KDEL-deleted variant. Both primers contain a BamH I restriction site (GGATCC) followed by stop codon as shown:

Wild-type:

5' TCG GAT CCC TAC AAC TCA TCT TTT TCT G 3'

[SEQ ID No. 24]

KDEL-deleted:

5' TCG GAT CCC TAT TCT GAT GTA TCC TCT TCA CC 3'

[SEQ ID No. 25]

The PCR products were cut with Msc I and BamH I and ligated into pET22 (Novagen) which had also been cut with these enzymes. The ligation product was used to transform competent BL21 cells. Clones obtained were screened by expression screening.

Expression and Purification

The procedure is identical for wild-type or KDEL-deleted BiP. Two liters of BL21 cells transformed with pET22 containing a sequence coding for either wild-type or KDEL deleted BiP were grown in 2xTY medium supplemented with 200μg/ml ampicillin at 37°C until they reached an absorbance at 600 nm of 0.5-0.6 at which point they were induced by the addition of 1 mM IPTG. The cells were allowed to grow for a further 2-5 hours at 37°C and then they were harvested by 10 minutes centrifugation at 7000 x G. The cell pellet was gently resuspended in 400 mL (or 80 mL/gm cells) of 30 mM Tris pH 8.0, 20% Sucrose, 1 mM PMSF. Following resuspension of the cells EDTA was added to 1 mM and the suspension

incubated at room temperature for 5 minutes. The cells were then spun down for 15 minutes at 7000 x G and resuspended in 400 mL of ice cold 5 mM $MgSO_4$, 1 mM PMSF and incubated at 4°C for 10 minutes. The cells were then spun down once again and the supernatant kept since this now constitutes the periplasmic extract.

The periplasmic extract was loaded onto a Pharmacia XK26 column containing 25 mL of DE52 anion exchange resin (Whatman) which had been equilibrated in 50 mM Mops pH 7.4, 10 mM NaCl. The bound protein was eluted in a 10-500 mM NaCl gradient. Fractions containing eluted BiP were identified by SDS-PAGE and pooled. The pooled BiP was subsequently run onto a Pharmacia XK26 column containing 10 mL of ATP agarose which had been equilibrated in 50 mM Mops pH 7.4., 100 mM NaCl, 5 mM MgAcetate, 10 mM KCl. After loading the pooled BiP containing fractions the column was washed until the baseline of absorption at 280 nm reached zero. Finally the bound BiP was eluted with the same buffer supplemented with 1 mM ATP. The eluate was concentrated by filtration, made up to 10% glycerol and stored frozen at -80°C.

EXAMPLE 13

PREPARATION OF RECOMBINANT MOUSE HSP40

Plasmid Constructions

The DNA fragment used to introduce an Nde I site at the initiation methionine of hsp40 was constructed via polymerase chain reaction (PCR) using an Nde-primer

5'-CCGCAGGAGGGGCATATGGGTAAAGAC-3' [SEQ ID No. 26]

and an Nco-primer

5'-GAGGGTCTCCATGGAATGTGTAGCTG-3' [SEQ ID No. 27].

The latter included an Nco I site corresponding to nucleotide 322 of the human hsp40 cDNA clone, pBSII-hsp40, Ohtsuka, K., *Biochem. Biophys. Res. Commun.* 197: 235-240 (1991), which was used as the template. The Hsp40-coding region of pBSII-hsp40 was digested with BamH I and Sac I and inserted into the complementary sites in a modified form of the plasmid pET-3a (Novagen, Inc.). The PCR-amplified DNA

was digested with Nde I and Nco I, and replaced the Nde I-Nco I region of the above plasmid to create the plasmid pET/hsp40, expressing hsp40.

Protein Purification:

To purify recombinant human hsp40, the plasmid pET/hsp40 was transformed into BL21(DE3) cells grown at 37°C. After a 2 hour incubation with 0.4 mM isopropyl thio-b-D-galactoside (IPTG), cells were lysed in a French Pressure Cell (SLM Instruments, Inc.) in buffer A [20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA] containing 1 mM PMSF. The cleared lysate was mixed with DEAE-Sephacel (Pharmacia) on ice for 1 h. The unbound material was collected and the resin was washed with buffer A. The flow-through and first wash were combined and loaded onto a hydroxyapatite HTP column (Bio-Rad) equilibrated with 100 mM potassium phosphate, pH 7.6. The column was washed with the same buffer and Hsp40 was eluted with a linear gradient of 100-300 mM potassium phosphate, pH 7.6. Peak fractions were rechromatographed on an HTP column after passing them through a DEAE-Sephacel column.

EXAMPLE 14

Vaccine compositions were prepared by combining recombinant mouse hsp70 (prepared as in example 2), recombinant human hsp40 (prepared as in example 13) and Ova-peptide

Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu [SEQ ID NO. 7]

in a final volume of 10 µl of buffer (20 mM Hepes pH 7.0, 150 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ and 2 mM MgATP) as follows:

Sample	hsp70	hsp40	ova
OVA-alone	nil	nil	5 ug
Hsp70/40	15 ug	8 ug	nil

Hsp70/40 + OVA	15 ug	8 ug	5 ug
Hsp70 + OVA	15 ug		5 ug

The mixtures were incubated for 30 minutes at 37°C prior to use for immunizations.

C57BL/6 mice were immunized intradermally once a week for a total of two weeks with 10 uL of one of the mixtures described above or with a mixture of TiterMax® (Vaxcell, Norcross, GA) and Ova-peptide (5 ug). One week after the second immunization, spleen cells were removed and mononuclear cells (6-8X10^7) were cultured with 3 X 10^6 γ-irradiated (3000 rad) stimulator cells. The stimulator cells were obtained from naive mice that had been sensitized *in vitro* with ova peptide (10 mg/ml) for 30 minutes at room temperature, washed and irradiated at 3000 rads.

Cytotoxicity of spleen cells from vaccinated mice was assayed on Ova-peptide pulsed EL4 cells in an 18-hour chromium release assay. CTL were generated by culturing *in vivo* immunized spleen cells for 5 days at a concentration of 10^6 cells/mL in RPMI medium, 10% FCS, penicillin, streptomycin and 2 mM L-glutamine, together with 3 X 10^6 γ-irradiated (3,000 rad) stimulator cells/mL. Target cells were prepared by culturing cells for 1 hour in the presence of 250 uCi of ^{51}Cr sodium chromate (DuPont, Boston, MA) in Tris-phosphate buffer, pH 7.4 at 37°C for 60 minutes. After washing, 10^4 ^{51}Cr-labeled target cells were mixed with effector lymphocytes to yield several different effector/target (E/T) ratio and were incubated for 18 hours. Supernatants were harvested and the radioactivity was measured in a gamma counter. Percent specific lysis was calculated as: 100 X [Cpm release by CTL- cpm spontaneous release)/ (cpm maximal release - cpm spontaneous release)]. Maximal response was determined by addition of 1% Triton X-100. Spontaneous release of all target in the absence of effector cells was less than 25% of the maximal release.

The results of this study are shown in Fig. 8. As shown, combinations of antigen with hsp70 or a mixture of hsp70 and hsp40 are effective to produce a CTL response to the antigen, while the administration of the antigen alone or a combination of heat shock proteins is not.

EXAMPLE 15

The experiment of Example 14 was repeated using EG7 lymphoma cells, Moore et al., *Cell* 54: 777-785 (1988), in place of the EL4 cells. The results are shown in Fig. 9 and are comparable to those observed using EL4 cells.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

CLAIMS

1. A composition for inducing a therapeutic immune response in a subject, comprising:

(a) a target antigen; and

(b) a heat shock protein;

wherein the target antigen and the heat shock protein are combined *in vitro* under conditions wherein binding of target antigen to heat shock protein occurs to form a target antigen/heat shock protein complex;

wherein the administration of the target antigen/heat shock protein complex to the subject induces an immune response comprising a cytotoxic cellular component.

2. The composition of claim 1, wherein the heat shock protein is hsp70.

3. The composition of claim 1, wherein the heat shock protein is gp96.

4. The composition of claim 1, wherein the heat shock protein is hsp40.

5. The composition of claim 1, wherein the heat shock protein is BiP.

6. The composition of any of claims 1 to 5, wherein the target antigen is a hybrid antigen.

7. The composition according to claim 6 wherein the hybrid antigen comprises an antigenic domain derived from a first source and a binding domain which binds to a heat shock protein from a second source different from the first source.

8. The composition of claim 7, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

9. The composition of claim 7, wherein the binding domain comprises a region having the sequence
His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]

10. A composition for inducing a therapeutic immune response in a subject, comprising:

(a) a nucleic acid molecule comprising a region encoding a target antigen operably linked to a promoter element; and

(b) a nucleic acid molecule comprising a region encoding a heat shock protein operably linked to a promoter element;

wherein the introduction of the nucleic acids of (a) and (b) into a cell result in the binding of target antigen to heat shock protein.

11. The composition of claim 10, wherein the nucleic acid molecules of (a) and (b) are comprised in the same vector.

12. The composition of claim 10 or 11, wherein the heat shock protein is hsp70.

13. The composition of claim 10 or 11, wherein the heat shock protein is gp96.

14. The composition of claim 10 or 11, wherein the heat shock protein is hsp40.

15. The composition of claim 10 or 11, wherein the heat shock protein is BiP.

16. The composition of any of claims 10 to 15, wherein the target antigen is a hybrid antigen.

17. The composition according to claim 16, wherein the hybrid antigen comprises an antigenic domain derived from a first source and a binding domain which binds to a heat shock protein. from a second source different from the first source.

18. The composition of claim 17, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

19. The composition of claim 17, wherein the binding domain comprises a region having the sequence His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]

20. A method of inducing an immune response in a subject in need of such treatment, comprising administering to the subject a therapeutically effective amount of the composition of any of claims 1 to 19.

21. A hybrid peptide comprising:

(a) an antigenic domain derived from a first source; and

(b) a binding domain which binds to a heat shock protein, said binding domain being derived from a second source different from the first source.

22. The hybrid peptide of claim 21, wherein the antigenic domain is derived from a virus, a parasite, a mycoplasma, a fungus or a bacterium.

23. The hybrid peptide of any of claims 21-22, wherein the antigenic domain elicits an immune response to a neoplastic disease.

24. The hybrid peptide of claim 23, wherein the neoplastic disease is selected from among a sarcoma, a lymphoma, a carcinoma, a leukemia and a melanoma.

25. The hybrid peptide of any of claims 21 to 24, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

26. The hybrid peptide of any of claim 21 to 24, wherein the binding domain comprises a region having the sequence

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1].

27. The hybrid peptide of any of claims 21 to 24, wherein the binding domain comprises at least a pentapeptide region selected from among

Gln Lys Arg Ala Ala [Seq. ID No. 5], and

Arg Arg Arg Ala Ala [Seq. ID No. 6].

28. A polynucleotide construct comprising:

(a) a region encoding a hybrid peptide comprising an antigenic domain derived from a first source; and a binding domain which binds to a heat shock protein said binding domain being derived from a second source different from the first source;

(b) a promoter effective to promote expression on the hybrid peptide in mammalian cells.

29. The polynucleotide construct of claim 28, wherein the antigenic domain is derived from a virus, a parasite, a mycoplasma, a fungus or a bacterium.

30. The polynucleotide construct of claim 28, wherein the antigenic domain elicits an immune response to a neoplastic disease.

31. The polynucleotide construct of claim 30, wherein the neoplastic disease is selected from among a sarcoma, a lymphoma, a carcinoma, a leukemia and a melanoma.

32. The polynucleotide construct of any of claims 28 to 31, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

33. The polynucleotide construct of any of claims 28 to 31, wherein the binding domain comprises a region having the sequence

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1].

34. The polynucleotide construct of any of claim s 28 to 31, wherein the binding domain comprises at least a pentapeptide region selected from among

Gln Lys Arg Ala Ala [Seq. ID No. 5], and

Arg Arg Arg Ala Ala [Seq. ID No. 6].

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(54) Title: HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES

(57) Abstract

The present invention relates to methods and compositions for inducing an immune response in a subject, wherein the subject is administered an effective amount of at least one heat shock protein in combination with one or more defined target antigens. These methods and compositions may be used in the treatment of infectious diseases and cancers.

CLAIMS

1. A composition for inducing a therapeutic immune response in a subject, comprising:

(a) a target antigen; and

(b) a heat shock protein;

wherein the target antigen and the heat shock protein are combined *in vitro* under conditions wherein binding of target antigen to heat shock protein occurs to form a target antigen/heat shock protein complex;

wherein the administration of the target antigen/heat shock protein complex to the subject induces an immune response to the target antigen comprising a cytotoxic cellular component.

2. The composition of claim 1, wherein the heat shock protein is hsp70.

3. The composition of claim 1, wherein the heat shock protein is gp96.

4. The composition of claim 1, wherein the heat shock protein is hsp40.

5. The composition of claim 1, wherein the heat shock protein is BiP.

6. The composition of any of claims 1 to 5, wherein the target antigen is a hybrid antigen.

7. The composition according to claim 6 wherein the hybrid antigen comprises an antigenic domain derived from a first source and a binding domain which binds to a heat shock protein from a second source different from the first source.

8. The composition of claim 7, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

9. The composition of claim 7, wherein the binding domain comprises a region having the sequence
His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]

10. A composition for inducing a therapeutic immune response to a target antigen in a subject, comprising:

(a) a nucleic acid molecule comprising a region encoding the target antigen operably linked to a promoter element; and

(b) a nucleic acid molecule comprising a region encoding a heat shock protein operably linked to a promoter element;

wherein the introduction of the nucleic acids of (a) and (b) into a cell result in the binding of target antigen to heat shock protein.

11. The composition of claim 10, wherein the nucleic acid molecules of (a) and (b) are comprised in the same vector.

12. The composition of claim 10 or 11, wherein the heat shock protein is hsp70.

13. The composition of claim 10 or 11, wherein the heat shock protein is gp96.

14. The composition of claim 10 or 11, wherein the heat shock protein is hsp40.

15. The composition of claim 10 or 11, wherein the heat shock protein is BiP.

16. The composition of any of claims 10 to 15, wherein the target antigen is a hybrid antigen.

17. The composition according to claim 16, wherein the hybrid antigen comprises an antigenic domain derived from a first source and a binding domain which binds to a heat shock protein. from a second source different from the first source.

18. The composition of claim 17, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

19. The composition of claim 17, wherein the binding domain comprises a region having the sequence His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]

20. A method of inducing an immune response to a target antigen in a subject in need of such treatment, comprising administering to the subject a therapeutically effective amount of the composition of any of claims 1 to 19, 35 or 36.

21. A hybrid peptide comprising:

(a) an antigenic domain derived from a first source; and

(b) a binding domain which binds to a heat shock protein, said binding domain being derived from a second source different from the first source.

22. The hybrid peptide of claim 21, wherein the antigenic domain is derived from a virus, a parasite, a mycoplasma, a fungus or a bacterium.

23. The hybrid peptide of any of claims 21-22, wherein the antigenic domain elicits an immune response to a neoplastic disease.

24. The hybrid peptide of claim 23, wherein the neoplastic disease is selected from among a sarcoma, a lymphoma, a carcinoma, a leukemia and a melanoma.

25. The hybrid peptide of any of claims 21 to 24, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

26. The hybrid peptide of any of claim 21 to 24, wherein the binding domain comprises a region having the sequence

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1].

27. The hybrid peptide of any of claims 21 to 24, wherein the binding domain comprises at least a pentapeptide region selected from among

Gln Lys Arg Ala Ala [Seq. ID No. 5], and
Arg Arg Arg Ala Ala [Seq. ID No. 6].

28. A polynucleotide construct comprising:

(a) a region encoding a hybrid peptide comprising an antigenic domain derived from a first source; and a binding domain which binds to a heat shock protein said binding domain being derived from a second source different from the first source;

(b) a promoter effective to promote expression on the hybrid peptide in mammalian cells.

29. The polynucleotide construct of claim 28, wherein the antigenic domain is derived from a virus, a parasite, a mycoplasma, a fungus or a bacterium.

30. The polynucleotide construct of claim 28, wherein the antigenic domain elicits an immune response to a neoplastic disease.

31. The polynucleotide construct of claim 30, wherein the neoplastic disease is selected from among a sarcoma, a lymphoma, a carcinoma, a leukemia and a melanoma.

32. The polynucleotide construct of any of claims 28 to 31, wherein the binding domain comprises at least a heptameric region having the sequence

HyXHyXHyXHy

where Hy represents a hydrophobic amino acid residue and X is any amino acid.

33. The polynucleotide construct of any of claims 28 to 31, wherein the binding domain comprises a region having the sequence

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1].

34. The polynucleotide construct of any of claim s 28 to 31, wherein the binding domain comprises at least a pentapeptide region selected from among

Gln Lys Arg Ala Ala [Seq. ID No. 5], and

Arg Arg Arg Ala Ala [Seq. ID No. 6].

35. The composition of any of claims 1 to 19, wherein the heat shock protein is a eukaryotic heat shock protein.

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36. The composition according to any of claims 1 to 19 or 35, wherein the target antigen is non-covalently bound to the heat shock protein.

37. A method of making a complex of a heat shock protein and a target antigen comprising combining a heat shock protein and a target antigen *in vitro* under conditions whereby reversible binding of th heat shock protein to the target antigen occurs.

38. The method of claim 37, wherein the target antigen is a hybrid antigen comprising an antigenic domain derived from a first source and a heat shock protein-binding domain derived from a second source different from the first source.

39. A complex of a heat shock protein and a target antigen made by the method co claim 37 or 38.

40. A composition comprising a complex made by the method of claim 37 or 38.

COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT or CIP Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES
the specification of which: *(complete (a), (b) or (c) for type of application)*

Regular or Design Application

(a) [X] is attached hereto.
(b) [ ] was filed on as Application Serial No. and was amended on *(if applicable).*

PCT Filed Application Entering National Stage

(c) [x] was described and claimed in International Application No.PCT/US96/13363 filed on August 16, 1996 and was amended on September 18, 1997.

Acknowledgment of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [X] such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
COUNTRY	APPLICATION NO	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/US96/13363	16/8/96		[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
				[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
60/002,490	August 18, 1995
60/002,479	August 18, 1995

Continuation-In-Part

(complete this part only if this is a continuation-in-part application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No) (Filing Date) (Status) (patented, pending, abandoned)

(Application Serial No) (Filing Date) (Status) (patented, pending, abandoned)

Power of Attorney

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POST OFFICE ADDRESS	POST OFFICE ADDRESS:	CITY:	STATE or COUNTRY:	ZIP CODE:
DATE	SIGNATURE OF INVENTOR			

Check proper box(es) for any added page(s) forming a part of this declaration

[ ] Signature for ninth and subsequent joint inventors. Number of pages added _____.

[ ] Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added _____.

[ ] Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47. Number of pages added _____.

FULL NAME OF SECOND JOINT INVENTOR, IF ANY	LAST NAME Hartl	FIRST NAME F.	MIDDLE NAME Ulrich	
RESIDENCE & CITIZENSHIP	CITY Munich	STATE or FOREIGN COUNTRY Germany	COUNTRY OF CITIZENSHIP Germany	
POST OFFICE ADDRESS	POST OFFICE ADDRESS Grasmeierstr. 22	CITY Munich	STATE or COUNTRY Germany	ZIP CODE 80805
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF THIRD JOINT INVENTOR, IF ANY	LAST NAME Hoe	FIRST NAME Mee	MIDDLE NAME H.	
RESIDENCE & CITIZENSHIP	CITY New York	STATE or FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 312 E. 66th Street	CITY New York	STATE or COUNTRY New York	ZIP CODE 10021
DATE Feb 10. 1998	SIGNATURE OF INVENTOR Mee H Hoe			
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	~~LAST NAME~~ Houghton	FIRST NAME Alan	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY New York	STATE or FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 1233 York Avenue, Apt 23B ~~402 E. 64th Street, Apt. 7C~~	CITY New York	STATE or COUNTRY New York	ZIP CODE 10021
DATE 2-10-98	SIGNATURE OF INVENTOR Alan Houghton			
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	LAST NAME Takeuchi	FIRST NAME Yoshizumi	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY New York	STATE or FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 1635 York Avenue, Apt. 24M	CITY New York	STATE or COUNTRY New York	ZIP CODE 10021
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	LAST NAME Mayhew	FIRST NAME Mark	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY Tarrytown	STATE or FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP Great Britain	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 414 Benedict Avenue, Apt. 3E	CITY Tarrytown	STATE or COUNTRY New York	ZIP CODE 10591
DATE 02/10/98	SIGNATURE OF INVENTOR Mark Mayhew			
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	LAST NAME	FIRST NAME	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
DATE	SIGNATURE OF INVENTOR			

Check proper box(es) for any added page(s) forming a part of this declaration

[ ] Signature for ninth and subsequent joint inventors. Number of pages added _____.

[ ] Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added _____.

[ ] Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47. Number of pages added _____.

BAKER & BOTTS L.L.P.
FILE NO.: 31567 PCT USA

COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT or CIP Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES
the specification of which: *(complete (a), (b) or (c) for type of application)*

Regular or Design Application

(a) [X] is attached hereto.
(b) [ ] was filed on as Application Serial No. and was amended on *(if applicable)*.

PCT Filed Application Entering National Stage

(c) [x] was described and claimed in International Application No.PCT/US96/13363 filed on August 16, 1996 and was amended on September 18, 1997.

Acknowledgment of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [X] such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
COUNTRY	APPLICATION NO.	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/US96/13363	16/8/96		[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]
ALL FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGNS) PRIOR TO SAID APPLICATION				
				[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]

BAKER & BOTTS, L.L.P.
FILE NO.: 31567 PCT USA

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
60/002,490	August 18, 1995
60/002,479	August 18, 1995

Continuation-In-Part

(complete this part only if this is a continuation-in-part application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

Power of Attorney

As a named inventor, I hereby appoint Dana M. Raymond, Reg. No. 18,540; Frederick C. Carver, Reg. No. 17,021; Francis J. Hone, Reg No. 18,662; Joseph D. Garon, Reg. No. 20,420; Arthur S. Tenser, Reg. No. 18,839; Ronald B. Hildreth, Reg. No. 19,498; Thomas R. Nesbitt, Jr., Reg. No. 22,075; Robert Neuner, Reg. No. 24,316; Richard G. Berkley, Reg. No. 25,465; Richard S. Clark, Reg. No. 26,154; Bradley B. Geist, Reg. No. 27,551; James J. Mume, Reg. No. 26,946; John D. Murnane, Reg. No. 29,836, Henry Tung, Reg. No. 29,705, Robert C. Scheinfeld, Reg. No. 31,300, John A. Fogarty, Jr., Reg. No. 22,348, Louis S. Sorell, Reg. No. 32,439 and Rochelle K. Seide Reg. No. 32,300 of the firm of BAKER & BOTTS, L.L.P., with offices at 30 Rockefeller Plaza, New York, New York 10112, as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
BAKER & BOTTS, L.L.P. 30 ROCKEFELLER PLAZA, NEW YORK, N.Y. 10112	BAKER & BOTTS, L.L.P. (212) 705-5000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF SOLE OR FIRST INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	Rothman	James	E.	
RESIDENCE & CITIZENSHIP	CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	New York	New York	US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
	402 E. 64th Street, Apt. 10B	New York	New York	10021
DATE	SIGNATURE OF INVENTOR			

FULL NAME OF SECOND JOINT INVENTOR, IF ANY	LAST NAME: Hartl	FIRST NAME: F.	MIDDLE NAME: Ulrich	
RESIDENCE & CITIZENSHIP	CITY: Munich	STATE or FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: Grasmeierstr. 22	CITY: Munich	STATE or COUNTRY: Germany	ZIP CODE: 0805
DATE: 2/10/98	SIGNATURE OF INVENTOR: F.U. Hartl			
FULL NAME OF THIRD JOINT INVENTOR, IF ANY	LAST NAME: Hoe	FIRST NAME: Mee	MIDDLE NAME: H.	
RESIDENCE & CITIZENSHIP	CITY: New York	STATE or FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 312 E. 66th Street	CITY: New York	STATE or COUNTRY: New York	ZIP CODE: 10021
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	LAST NAME: Houghton	FIRST NAME: Alan	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: New York	STATE or FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 402 E. 64th Street, Apt. 7C	CITY: New York	STATE or COUNTRY: New York	ZIP CODE: 10021
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	LAST NAME: Takeuchi	FIRST NAME: Yoshizumi	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: New York	STATE or FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: US	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 1635 York Avenue, Apt. 24M	CITY: New York	STATE or COUNTRY: New York	ZIP CODE: 10021
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	LAST NAME: Mayhew	FIRST NAME: Mark	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: Tarrytown	STATE or FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: Great Britain	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 414 Benedict Avenue, Apt. 3E	CITY: Tarrytown	STATE or COUNTRY: New York	ZIP CODE: 10591
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	LAST NAME	FIRST NAME	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
DATE	SIGNATURE OF INVENTOR			

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[ ] Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added _____.

[ ] Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47. Number of pages added _____.

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

POWER OF ATTORNEY BY ASSIGNEE OF ENTIRE INTEREST (REVOCATION OF PRIOR POWERS AND APPOINTMENT OF NEW POWER)

Docket Number.
11390/1

Application Number	Filing Date	Examiner	Art Unit
09/011,645	February 13, 1998	T. Worrall	1642

Invention Title	Inventor(s)
HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES	Rothman et al.

Address to:
Assistant Commissioner for Patents
Washington D.C. 20231

As assignee of the entire interest of the above-identified application by virtue of an executed Assignment, recorded in the U.S. Patent and Trademark Office on March 23, 1998, under Reel 9051, Frame 0954, all powers of attorney previously given are hereby revoked and the following attorneys and/or agents are hereby appointed to prosecute and transact all business in the Patent and Trademark office connected therewith:

Richard L. DeLucia (Reg. No. 28,839)
Donna M. Praiss (Reg. No. 34,232)
Charles A. Weiss (Reg. No. 40,867)
Christine M. Wilkes (Reg. No. 37,967)
Houri Khalilian (Reg. No. 39,546)
M. Lisa Wilson (Reg. No. 34,045)

SEND CORRESPONDENCE, AND DIRECT TELEPHONE CALLS TO:

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Sloan-Kettering Institute for Cancer Research

Date: 1/26/00

By: James S Quirk

Name: James S. Quirk
Title: Senior Vice President
Research Resources Management